Influences of the First-feeding Delay on Subsequent Growth and Viability of Clonal Crucian Carp, *Carassius langsdorfii*

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Abstract   Clonal nature of gynogenetic progeny of triploid crucian carp *Carassius langsdorfii*, was verified by multilocus DNA fingerprinting techniques. Clones were used to assess the effects of first-feeding delay on subsequent growth and viability.

On the 3rd day after hatching, the typical larvae having available yolk commenced the first-feeding. On the 4th day, total length of unfed larvae for 1 day was significantly shorter than that of fed larvae. There was no difference in dry weight between fed and unfed larvae. The difference in the RNA:DNA ratio between fed and unfed larvae became evident on Day 4.

The results of communal stocking of both fed and delay-fed larvae for 1 to 2 day revealed that the growth loss caused by the delay-fed treatments was not compensated through subsequent growth. However, it was evident that survival rates in delay-fed larvae for 2 day slightly higher than non delay-fed larvae. This result can be explained by the selection of some larvae hatched with developmental defects during delay-fed treatment.

Key words: crucian carp, clone, first-feeding delay, growth, otolith-tagging, PNR

INTRODUCTION

The transition from endogenous nutrition to exogenous feeding is important in survival potential of post-embryonic stage of many fish species. High mortality of fish larvae just after absorption of the yolk is generally attributed to starvation caused by a mismatch between food quality and food requirements (HJORT, 1914; HUNTER, 1976). Since Hjort (1914) proposed the concept of the "critical period" in the early life stage of fish, considerable efforts have been done to distinguish starved larvae from non-starving larvae by morphological (EHRLICH *et al.*, 1976; NEILSON *et al.*, 1986; STRUSSMANN and TAKASHIMA, 1989), histological (O'CONNELL, 1976; THEILACKER and WATANABE, 1989, STRUSSMANN and TAKASHIMA, 1989; MCFADZEN *et al.*, 1994) and biochemical criteria (EHRLICH, 1974; BUCKLEY, 1979; CLEMMESEN, 1994; SATO *et al.*, 1995; KIMURA *et al.*, 1996). Consequently, nutritional condition of larvae has been recognized as the most important factor in determining individual viability. However, we have little information about the influences of the first-feeding delay on subsequent survival and growth.

One of the reason why such a kind of study is limited, is an absence of experimental

animal with isogenic characters. Differences in genetic background within the materials often makes the result inconclusive. Genetic potential for growth or survival varies among fish stock (VINCENT, 1960; UCHIDA *et al.*, 1989) and even within siblings (MAGNUSON, 1962). Although, artificial cloning of fish has been experimentally succeeded by the chromosome manipulation for a few species such as ayu *Plecoglossus altivelis* (HAN *et al.*, 1991), carp *Cyprinus carpio* (KOMEN *et al.*, 1991), hirame flounder *Paralichthys olivaceus* (HARA *et al.*, 1993) and amago salmon *Oncorhynchus rhodurus* (KOBAYASHI *et al.*, 1994). It is generally difficult to obtain isogenic lines for routine nutritional studies. On the other hands, unisexual fish species which reproduce clonally in nature are considered useful as the experimental animal because of no genetic variance within a strain. Crucian carp, *Carassius langsdorfii*, are widely distributed in Japan where they are known as ginbuna, and includes triploid forms (KOBAYASHI, 1971; ONOZATO, 1981; ONOZATO *et al.*, 1983; NAKAKUNI *et al.*, 1984). The triploid ginbuna is unisexual and lays unreduced triploid eggs (KOBAYASHI, 1976; YAMASHITA *et al.*, 1993). They reproduce by gynogenesis; insemination by sperm of diploid ginbuna or of other species initiates the development of the triploid ginbuna eggs. However, the incorporated sperm nucleus does not transform into a male pronucleus and makes no contribution to the zygotic genome (KOBAYASHI, 1971; OJIMA and ASANO, 1977; NAKAKUNI *et al.*, 1984; YAMASHITA *et al.*, 1990). Therefore, all progeny from a triploid crucian carp are members of a genetically identical clone that contains only maternal chromosomes and are expected to be a clonal line in nature (ONOZATO, 1981; MURAYAMA *et al.*, 1984). UMINO *et al.* (1997) reported that the triploid crucian carp in the Kurose River are categorized into a few clonal families and the progeny from each clonal family was generated.

The aims of this study were twofold. First, we verified the clonal nature of gynogenetic progeny of triploid crucian carp from the Kurose River using multilocus DNA fingerprinting. Second, we assessed the effects of first-feeding delay on subsequent growth and viability. In addition, the results of "point-of-no-return" (PNR) studies are also presented.

MATERIALS AND METHODS

Production of gynogenetic progeny

Triploid ginbuna were obtained by fishing from the Kurose River, Higashi-hiroshima city, Hiroshima Prefecture, in the spring of 1994. Triploidy was initially assessed based on erythrocytic size; triploid have significantly larger erythrocytic diameters than diploid of *Carassius* species (SEZAKI *et al.*, 1977; ONOZATO *et al.*, 1983). Fish was artificially ovulated by injecting human chorionic gonadotropin (10IU/g. fish). Eggs inseminated with heterospecific spermatozoa of the common carp, *Cyprinus carpio*, were scattered on the incubation containers (stocking density: 0.5 egg/cm^2). During the incubation, about half volume of the water in each incubation container was replaced with fully aerated water, and dead eggs were removed every day. About 5000 larvae hatched within 66 to 90h after insemination at 20.9±1.1°C. The larvae hatched 76 to 78h after insemination were used for the experiments (around 50% hatching).

PNR study

For the monitoring of mortality from hatching to death of starving larvae, larvae were

transferred carefully by wide-bore glass pipette from the incubation containers to three one-liter glass beakers (100 larvae counted into each).

On the other hand, the larvae stocked reservoir containers, giving a density of approximately 100^{-L} were used for re-feeding trials, daily samples of length measurement, and samples for biochemical analysis. For daily measurements of length, a minimum of 30 larvae were randomly taken from each container. For re-feeding trials at 3- to 11-d post hatch, 30 larvae were transferred by pipette from reservoir containers to one-liter glass beakers. During re-feeding trials, *Artemia* nauplii were added to the rearing beakers in excess four times a day for 10 days. During both starvation tests and re-feeding trials, oxygenated water was provided by replacing one half volume of the water in beakers with fresh water every second day. Water temperature ranged form 19.3 to 22.7°C (20.9±0.9°C, mean and S.D.).

Experimental design and effects of first-feeding delay

Larvae of teleost fish are generally considered to be the stage most sensitive to environmental stressors (Von WESTERNHAGEN, 1988). Temporal differences in environmental conditions among the rearing tanks may have a substantial effect on future growth and survival. To circumvent the problems of experimental errors due to tank-to-tank differences, we used the "communal stocking" concept developed by MOAV and WOHLFARTH (1976) and WOHLFARTH and MOAV (1985) to assess the effects of first-feeding delay on the subsequent growth and viability.

Newly hatched larvae were allotted to two treatment groups, non delay-fed as a control and delay-fed treatment for the first-feeding. For stock identification, the control larvae were tagged their otoliths according to TSUKAMOTO (1988) with slight modification. Larvae were treated with alizarin complexone (ALC) solution (7.5mg/l) for 12h. In a preliminary experiment with 50 tagged and 50 non-tagged larvae reared for 30 days under communal stocking, tagging gave no effect on growth and survival. On completion of the otolith-tagging, larvae were divided randomly into four rearing tanks (tank#1−4, 30×20cm, 4cm in depth), each with 50 fish. From the time of mouth-opening, freshly hatched *Artemia* spp. were added in excess to the tanks four times a day (9:00, 12:00, 14:00, 17:00). All larvae succeeded in the first-feeding until 3 days after hatching.

On Day 4, 50 larvae whose first-feeding had been delayed one day were added to the each of tanks#1 and #2. On Day 5, 50 larvae whose first-feeding had been delayed two days were added to tanks#3 and #4. Rearing lasted 30 days. Tanks were kept free of dead larvae or feeding residuum throughout the duration of the experiment. During the 30-d experiment, *Artemia* sp. nauplii were the sole food source. Aerated water was provided by replacing one half volume of the water in rearing tanks with fresh water every second day. Water temperature ranged 19.3 to 22.7°C (21.0±0.9°C, mean and S.D.) At the end of the experiment, total lengths and weights of all the fish were measured, and sagittae were removed and examined microscopically under UV light for stock identification. Larvae with ALC-tagging in the otoliths (Fig. 1) were regarded as the control group and those without, as the delay-fed group.
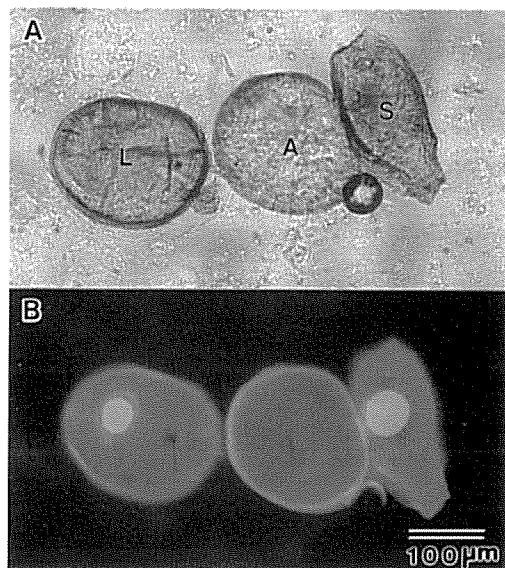
Fig. 1   ALC-tagging in the otoliths of crucian carp, *Carassius langsdorfii*.
         (A) Microscopic photograph of otolith on the 20th day after hatching.
         L; lapillus, A; asteriscus, S; sagitta.
         (B) The ALC-tagging UV light microscopic photograph of the same otoliths at the top.
         Newly hatched larvae treated with ALC have fluorescent-marks in both sagitta and lapillus.

DNA-fingerprinting

DNA was extracted from 50µl of blood from female, and from the entire body of juveniles of their progeny. Samples were taken into 400µl extraction buffer containing 10mM Tris-HCl (pH7.5), 125mM NaCl, 10mM EDTA, 0.5% SDS and 4M Urea (FUJIKAWA *et al.*, 1993). Proteinase K (50µg/ml) was added to the buffer and samples incubated at 37°C overnight. The purification of DNA was carried out in two successive extractions by standard techniques using salt-saturated phenol and chloroform.

Purified DNA was digested with *Hinf* I and/or *Hae* III (Takara Shuzo Co., Ltd., Japan). The fragments were electrophoresed in TAE (Tris-acetate-EDTA) buffer using 0.8% agarose gel at constant 50V for 24h or more. The gel was capillary blotted onto a nylon membrane. The blotted membrane was baked at 80°C for 2h. Non-isotopic multilocus DNA probe kits (NICE[TM], Jeffreys' 33.6 and/or 33.15, Cellmark Diagnostics, UK) were used for DNA fingerprinting. The membrane was prehybridized in 2×SSC, 2% SDS, 1% casein for 60min at 50°C and hybridized by probe (10µl) in 10ml 5×SSC, 2% SDS, 10% polyethylene glycol for 1h at 43°C. It was washed in 1×SSC, 1% SDS for 10min at 48°C, in 1×SSC, 1% SDS for 10min at 48°C and then 1×SSC for 10min at room temperature. This was followed by spraying with Lumi-phos 530[TM] and exposure to X-ray film for 2h.

Determination of dry weight and RNA:DNA ratio

Larval dry weight and nucleic acid analyses were performed on a pooled group of 50 and 3 larvae, respectively. For measurement of larval dry weight, samples were placed on

a weighting bottle, rinsed with distilled water, oven-dry at 60°C for 6h and 105°C for 15h, and stored in a desiccator. For extraction of nucleic acids, larvae were taken into 400μl extraction buffer and the nucleic acid was extracted twice with salt-saturated phenol and chloroform (see the *DNA fingerprinting* section). RNA and DNA contents was determined according to CLEMMESEN (1993). In brief, fluorescence-photometric measurement using a specific nucleic acid fluorescent dye, ethidium bromide was used for determination of nucleic acid contents. For determination of DNA content, RNA was digested by RNase (AMRES-CO Inc., USA) and the remaining DNA content was determined using salmon sperm DNA (Wako Pure Chem. Co., Ltd., Japan) and yeast RNA (Kanto Chem. Co., Ltd., Japan) as a standard.

Statistics

Total length, dry weight and RNA:DNA ratio of fed and unfed groups were examined by ANOVA. When the F-ratio for the ANOVA was statistically significant ($p < 0.05$), these values were further compared using Fisher's Protected Least Significant Difference (PLSD) test. Mean total lengths and body weights of delay fed and non delay fed groups after communal stocking for 30 days were compared by the t-test (equal variances) or by Welch's approximative t-method (unequal variances). The null hypothesis (H_o: $\mu_A = \mu_B$) was rejected at 0.05. For all statistical analyses, the Stat View^{TM} (Brain Power Inc., USA) program was used.
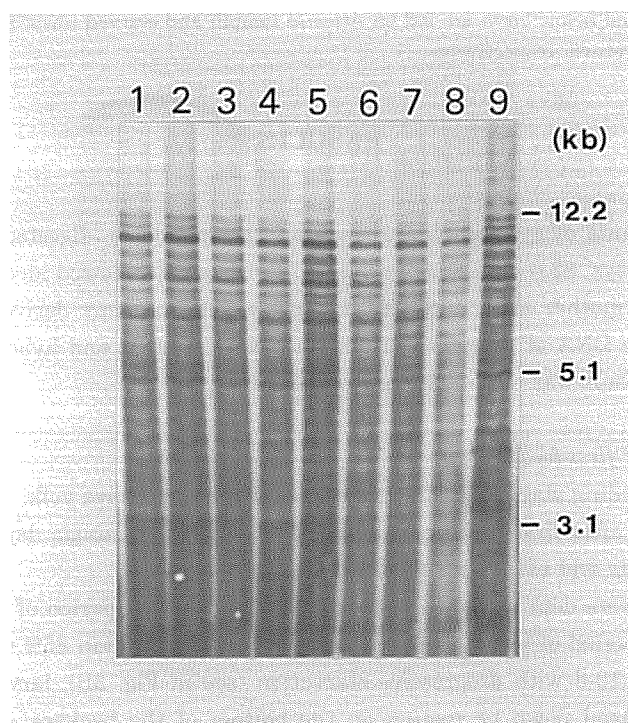

Fig. 2   DNA-fingerprints of gynogenetic progeny of crucian carp, *Carassius langsdorfii*. DNA was digested with *Hinf* I and hybridized to the Jeffreys' 33.6 probe. Lanes #1–8 and #9 are the progeny and their mother, respectively. DNA size markers are shown at the right. Molecular weight are given in base pair.
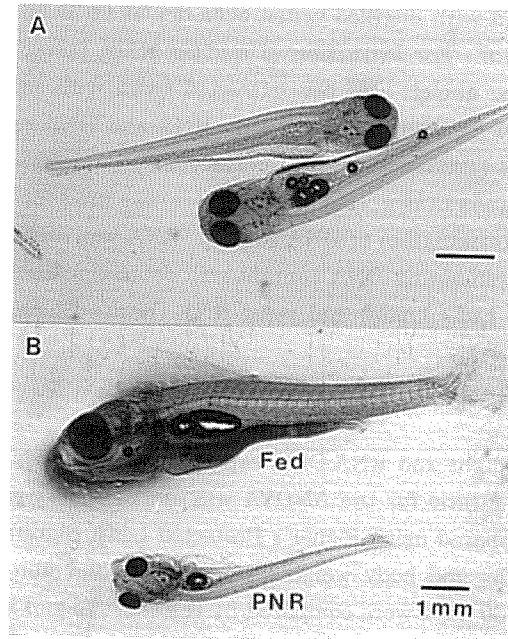
Fig. 3   Larvae of crucian carp, *Carassius langsdorfii*, have 3 and 11th day after hatching.
(A) Larvae immediately after the first-feeding still have availavle yolk.
(B) Above and below larva are fed on *Artemia* nauplii and starved toward to point-of-no-return, respectively.

RESULTS

Clonal nature of gynogenetic progeny

DNA fingerprints of gynogenetic progeny and their mother, digested with *Hinf* I and hybridized to Jeffreys' 33.6 are shown in Fig. 2. Fingerprints showed identical patterns not only between the mother and daughters but also among progeny derived from the same mother. When the DNA of progeny were digested with *Hae* III and hybridized to Jeffreys' 33.15, the same result was obtained (data not shown).

PNR and effects of first-feeding delay

At the first feeding stage (Fig. 3A), the typical larvae still have yolk. The yolk can also be abserved a day after first feeding. Therefore, larvae have a single day of mixed-feeding on both endogenous and exogenous feeding.

When larvae were deprived of food, there were two distinct period of mortality (Fig. 4). During an initial period of low mortality from 0- to 10-d, more than 85% of larvae survived. Beginning around 10-d with progressive starvation (see in Fig. 3B), larvae had symptoms such as hanging head down or resting on the bottom of the beakers, and then survival declined rapidly (Fig. 4).

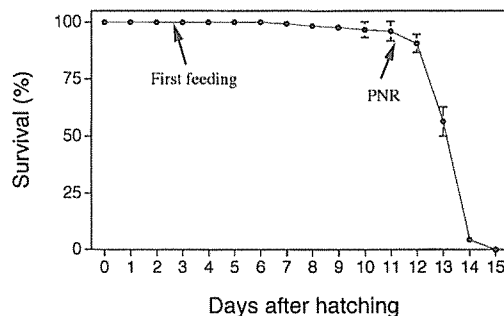The survival curve in re-feeding trials up to for 10-d after being delayed in first-feeding

Fig. 4   Starvation curve of newly hatched larvae crucian carp *Carassu langsdorfii* at 20.9 °C. Data are shown as mean±SD.
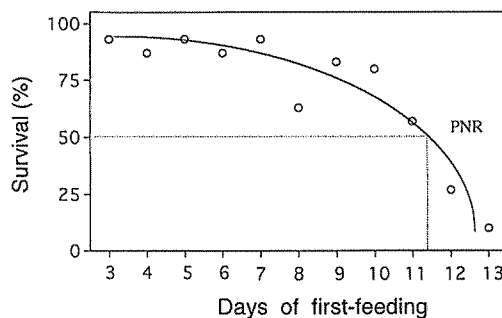
Fig. 5   Survival of crucian carp larvae treated with delayed-feeding for 0- to 10-d. Survival percentages were presented at the end of re-feeding trials for 10 days.

is illustrated in Fig. 5. Among the delay-fed groups from 1- to 4-d, the survival rates were almost constant level at 90%, similar to the control group. Following 5-d of delay-fed treatment, the survival gradually declined with longer delay-fed treatments. PNR, the maximum delay from which 50% of re-fed larvae can survive, was estimated to be 11.3-d post-hatch (Fig. 5).

Total length, dry weight and RNA:DNA ratio of the larvae fed *ad libitum* on *Artemia* nauplii for 1- to 2-d (F1 and F2 group, respectively) and those of the larvae experiencing unfed for 1- to 2-d (S1 and S2 group, respectively) are shown in Fig. 6. Larval total length in both F1 and S1 groups increased significantly compared with that of the first-feeding stage of 3-d. On Day 4, however, there was significant difference in mean total length between them due to relatively high total length gain of F1 group. The total length in F2 group showed progressively increased, although that of S2 was unchanged (Fig. 6A).

In terms of dry weight, there was no increase in the F1 group in comparison with the first-feeding stage. Moreover, no significant differences were found whether larvae were fed or starving. By Day 5, larval dry weight had significantly increased for F2 but had decreased for S2 (Fig. 6B).

The RNA:DNA ratio of the F1 group increased significantly compared with that of the
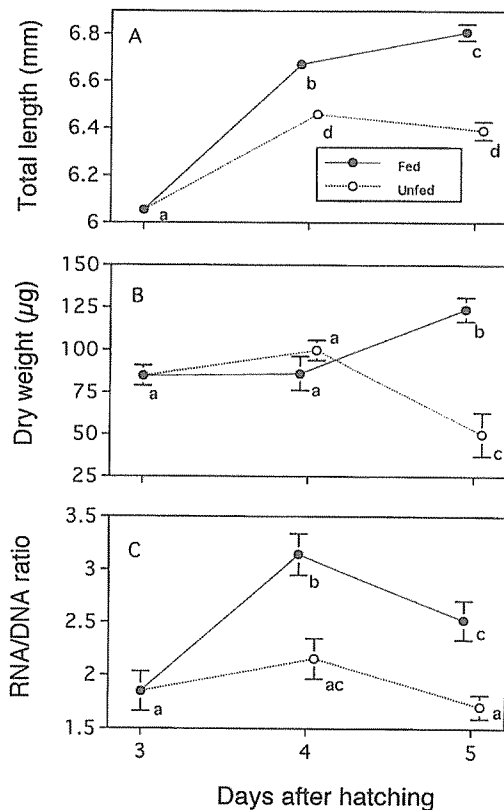
Fig. 6   Total length (A), dry weight (B) and RNA/DNA ratio (C) of the larvae fed *ad libitum* on *Artemia* nauplii or starved for 1 to 2 days after the first-feeding.   Data are shown as mean±SE. Different characters indicate significant differences at $p < 0.05$ level (Fisher's PLSD test).

first-feeding stage.   The ratio for the S1 group remained at the level of the first feeding group.   As a result, significant differences in the ratio of fed and unfed groups were evident beginning on Day 4, and could be seen thereafter (Fig. 6C).

Table 1 summarizes the influences of the first-feeding delay for 1- to 2-d on subsequent growth and viability after communal stocking for 30 days.   Total length and body weight of delay-fed 1-d group were significantly different in comparison with those of controls in both rearing tanks#1 and #2.   However, there were no differences in survival between larval groups after 30 d post-hatch.   Identical results were obtained in the tank#3 and #4 where delay-fed 2-d and control groups were stocked.   However, there was a tendency for survival rates of delay-fed 2-d groups to be higher than those of control groups.

DISCUSSION

Gynogenetic progeny of crucian carp have been considered to contain only maternal chromosomes identical to those of the somatic cells of the mother and are genetically identical clones (ONOZATO, 1981; MURAYAMA *et al.*, 1984) except for the occasional of partial synapsis and recombination (MURAYAMA *et al.*, 1984; OSAWA *et al.*, 1984; ZHANG *et al.*, 1992). Clonal strains are recognized in nature using scale-graft and protein electrophoresis

Table 1   Effects of the first-feeding delay for 1- to 2-d on subsequent growth and viabilty of crusian carp at 30th day after hatching

Component of raring tanks		Control	Delay-fed	t-test
Control vs Delay-fed 1 day				
Tank#1	Total length (Mean±S.D., mm)	13.4±0.54 (n=47)	13.0±0.60 (n=47)	p=0.0014
	Body weight (Mean±S.D., mg)	20.4±3.62 (n=47)	17.8±3.36 (n=47)	p=0.0003
	Survival (%)	94	94	
Tank#2	Total length (Mean±S.D., mm)	13.9±0.98 (n=47)	13.2±0.96 (n=45)	p=0.0019
	Body weight (Mean±S.D., mg)	22.3±5.02 (n=47)	19.3±5.17 (n=45)	p=0.0037
	Survival (%)	94	90	
Control vs Delay-fed 2 day		Control	delay-fed 2 day	
Tank#3	Total length (Mean±S.D., mm)	14.0±0.75 (n=43)	13.4±0.74 (n=47)	p<0.0001
	Body weight (Mean±S.D., mg)	25.5±4.77 (n=43)	21.4±4.13 (n=47)	p<0.0001
	Survival (%)	86	94	
Tank#4	Total length (Means±S.D., mm)	14.3±0.68 (n=41)	13.3±0.61 (n=47)	p<0.0001
	Body weight (Mean±S.D., mg)	25.9±4.52 (n=41)	19.2±3.40 (n=47)	p<0.0001
	Survival (%)	82	94	

(ONOZATO, 1981; MURAYAMA *et al.*, 1984) and DNA fingerprinting (UMINO *et al.*, 1997). In the present study, DNA fingerprinting techniques clearly demonstrated the clonal nature of progeny form the triploid crucian carp *Carassius langsdorfii* in the Kurose River. This result suggests that gynogenetic progeny is quite useful experimental animal in many aspects of biology because of isogemic nature. Isogenic lines of triploid crucian carp were used to examine environmental factors on variances in growth (NAKANISHI and ONOZATO, 1987) as well as cellular immune mechanisms (NAKANISHI, 1987).

It is noteworthy that larval total length exhibited significant increases even if their first feeding were delayed one day. The increasing of total length in delay fed larvae can be partly explained by consumption of available yolk. However, length gains of fed larvae was superior to delay-fed larvae. This suggests that exogenous feeding at the first-feeding stage plays an important role in length gain, and complements endogenous feeding.

Larval body weight remains almost constant for several days after the first-feeding in winterflounder, *Pseudopleuronectes americanus* (CETTA and CAPUZZO, 1982) and walleye pollock, *Theragra chalcogramma* (YAMASHITA and BAILEY, 1989), although daily food intake per body weight is maximal (STEPIEN, 1976; THEILACKER, 1987; YAMASHITA and BAILEY, 1989). In this study, larval dry weight showed no significant increase after feeding or starving for one day. Therefore, this phenomenon may be a general characteristic of innate larval physiology.

We confirmed by communal stocking that the growth loss due to delaying the first-feeding was not compensated through subsequent growth. In other words, initial size difference formed in first-feeding stage were maintained thereafter. However, FUKUHARA (1974), who studied influence of the first-feeding delay of red sea bream, *Pagrus major*, reported no remarkable difference in subsequent growth. A similar result was reported by RIBEIRO and PLANAS (1995) in turbot *Scophthalmus maximus* larvae. RIBEIRO and PLANAS (1995) explained that "catch-up growth" (WEATHERLY and GILL, 1981; MIGLAVS and JOBLING, 1989; PEDERSEN *et al.*, 1990) may contribute after the switch from starvation to feeding. On the contrary, the growth loss due to delaying the first-feeding was not compensated in subsequent growth in Pacific herring, *Clupeus harengus pallasi* (McGurk, 1984), turbot, *Scophthalmus maximus* (MCFADZEN *et al.*, 1994), and northern anchovy, *Engraulis mordax* (THEILACKER and WATANABE, 1989). Our results provide support for the latter results.

The RNA:DNA ratio of fed and unfed larvae could be clearly distinguished immediately after the first feeding. These results are concordant with earlier observations in Atlantic cod, *Gadus morhua* (BUCKLEY, 1979) and sardine, *Sardinops melanostictus* (SATO *et al.*, 1995; KIMURA *et al.*, 1996). Generally, teleost larvae at the first-feeding stage are endowed with basic structures of digestive organs such as gut, liver, and pancreas (TANAKA, 1971). The immediate development of these organs for subsequent exogenous feeding must take place. On the contrary, when larval first-feeding was deprived even in one day, histological degeneration, especially in the digestive tract was reported in pejerry, *Odontesthes bonariensis* by STRUSSMANN and TAKASHIMA (1989). MCFADZEN *et al.*, (1994), working on turbot larvae, *Scophthalmus maximus*, reported that atrophy of the digestive epithelium and regions of microvilli loss followed one day of first-feeding delay. Differences in RNA:DNA ratio between fed and unfed larvae were attributed to variations in the relative amounts of active cells. Thus differences have the potential to act for productive ability of the digestive enzymes, and a determining factor of subsequent larval growth as well as development of the digestive tract (THEILACKER and WATANABE, 1989).

We estimated that PNR of crucian carp was 11.3-d post-hatch at 20.9°C. This finding coincided with results from common carp suggested by histological diagnosis (Wang *et al.*, 1983). On the other hand, the starvation curve for crucian carp *Carassius langsdorfii* were characterized by an initian period of low mortality followed by mass mortality. The pattern is similar to those reported by CHIBA (1961) for common carp. Larval death in the initial period was considered due to genetic lethals and/or some larvae that hatched with developmental defects (FUKAHARA, 1974). In this study, larval death in the initial period may be attributed to the latter factor due to clonal effects of the materials. BLAXTER (1975) pointed out that hatchery rearing conditions permit a greater number of poor quality larvae to survive due to lower selection pressure. FUKUHARA (1974) reported that a first-feeding delay less than two days may exert a selective pressure on poor quality larvae of red sea bream, *Pagrus major*. The present results in survival ratio of larvae delay-fed for 2 days partly supports his views.

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クローンギンブナの初期摂餌開始時期が成長および生残に及ぼす影響

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　広島県黒瀬川由来の三倍体ギンブナー腹仔のクローン性をDNAフィンガープリント法によって確認した。それらのクローン仔魚を用いて初期摂餌開始時期の遅延が, 後の成長および生残に及ぼす影響を調べた。ギンブナ仔魚は孵化後3日目で初期摂餌を開始した。孵化後4日目における摂餌を1日間遅らせた群の全長は摂餌対照群のそれに比べて有意に小さかったが, 乾燥重量は摂餌の有無に関わらず変化が認められなかった。摂餌群と摂餌遅延群の核酸比は, 孵化後4日目で有意差が認められた。仔魚の初期摂餌を1日および2日遅らせた群に対し, それぞれの給餌対照群を混合し, 30日間飼育した。その結果, 摂餌を遅らせた群の平均全長および体重は対照群のそれより有意に小さくなった。したがって, 初期摂餌を遅らせることによって生じた給餌対照群との成長差は, その後も維持されることが明らかとなった。一方, 30日後の生残率では, 初期摂餌を2日間遅延した仔魚で高くなった。これは, 初期摂餌を遅延する間に, 質的に劣る仔魚が淘汰されたためと考えられた。
キーワード：ギンブナ, クローン, 初期摂餌, 成長, 耳石標識, PNR